Table 1: Summary of targeting experiments

Supplemental Figure 1: Surveyor (CEL-I) Nuclease Assay for OCT4 and AAVS1 ZFN pairs

A. Screening of ZFNs directed against the human *OCT4* locus. The indicated ZFNs (Supplemental Table 1) were transiently transfected into the cell types indicated, genomic DNA was isolated 48 hrs later, and the frequency of target locus disruption was measured by the Surveyor/Cel-1 assay using ^{32}P-labeled PCR exactly as described [26]. The frequency of gene disruption is indicated in each gel panel; "G" indicated cells transfected with an eGFP-expression vector as a control.

B. BGO1 hESCs were electroporated with an eGFP expression plasmid and the indicated ZFN pairs. As a control cells were electroporated without ZFN. GFP positive cells were isolated by FACS sorting 48 hours after electroporation. DNA was isolated and analyzed for the disruption of the *OCT4* and AAVS1 locus.

Supplemental Figure 2: FACS analysis of OCT4 targeted clones

FACS analysis of BGO1 cells and BGO1 cells targeted with the indicated OCT4 ZFN pairs. All cells were co-stained and analyzed for SSEA4 expression to exclude SSEA4 negative differentiated cells from the analysis.

Supplemental Figure 3: Southern blot analysis of hESCs targeted in the AAVS1 locus

A. Representative Southern blot results of BGO1 clones targeted to the AAVS1 locus using the AAVS1-SA-Puro donor plasmid. Southern blot analysis were performed as in Figure 2B. Correctly targeted clones are indicated in red. Similar to the OCT4 targeting discussed above, a fraction of clones, although targeted, carried additional integrations. The bottom panel shows the analysis of genomic DNA of a selecion of the clones shown above digested with EcoRV and probed with the external 3' probe. As, EcoRV does not cut in the donor vector the detection of higher than expected molecular weight restriction fragments in clone #3,7,8 and 10 suggests that the additional integration detected with the internal 5'-probe (middle panel) represent multiple integrations of the donor plasmids in the AAVS1 locus rather than an integration caused by off-target effects of the AAVS1 ZNFs. These clones were considered as not correctly targeted

and not analyzed further, since the majority of the clones obtained were correctly targeted on one or both ZFN-targeted alleles, and lacked randomly integrated DNA.

B. Southern blot analysis as in (A) from targeting experiments with the AAVS1-PGK-Puro donor plasmids.

Supplemental Figure 4: Characterization of BGO1 cells targeted in the AAVS1 locus

A. Representative Karyotype analysis of BGO1 cells correctly targeted in the AAVS1 locus using the AAVS1-PGK-Puro donor plasmid.

B. Immunofluorescence staining of BG01 cells correctly targeted with the indicated ZFN pairs using the corresponding donor plasmids. Cells were stained for the pluripotency markers OCT4, NANOG, SOX2, Tra-1-60 and SSEA4.

C. Hematoxylin and eosin staining of teratoma sections generated from BG01 cells targeted with the indicated ZFN pairs and the corresponding donor plasmids.

Supplemental Figure 5: ZFN mediated gene targeting of hiPSCs

A. Southern blot analysis of hiPSC cell line PD2^{1lox}-17Puro-5 targeted with the AAVS1 ZFN pairs using the indicated donor plasmids. Genomic DNA was digested with SphI and hybridized with the ^{32}P-labeled external 3' probe or with the internal 5' probe. Fragment sizes are: PGK-Puro: 5'-probe: wt=6.5 kb, targeted=4.2 kb; 3'-probe: wt=6.5 kb, targeted=3.7 kb. SA-Puro: 5'-probe: wt=6.5 kb, targeted=3.8 kb; 3'-probe: wt=6.5 kb, targeted=3.7 kb

B. Hematoxylin and eosin staining of teratoma sections generated from hiPSC cell line PD2^{1lox}17Puro-5 cells targeted with the AAVS1 ZFN pairs using the AAVS1-PGK-Puro donor plasmid.

Supplemental Figure 6: Time course experiment of DOX withdrawal of hESCs targeted with a TetO-eGFP cassette in the AAVS1 locus

FACS analysis of BGO1 cells either heterozygous or homozygous for the TetO-eGFP bw donor targeted to the AAVS1 locus. Prior to the experiment cells were transduced with a lentivirus carrying the M2rtTA reverse transactivator, DOX induced and subsequently enriched for eGFP expressing cells by FACS sorting. The graph shows the relative GFP expression of this cell population at the indicated times after DOX withdrawal. All cells were co-stained and analyzed for SSEA4 expression to exclude SSEA4 negative feeder cells from the analysis.

Supplemental Figure 7: Surveyor (CEL-I) Nuclease Assay for PITX3 ZFNs

Two distinct ZFN pairs were tested for disruption of the *PITX3* locus in K562 cells as described above. The more active pair (ZFN pair#2) was used for genome editing in hESCs and hiPSCs.

Supplemental Figure 8. Experimental evaluation of genotypes in single-cell derived clones heterozygous for a ZFN-driven targeted integration event at the AAVS1 locus.

A. Table of off-target sites identified as detailed in Supplementary Methods. “FTA” indicates loci that failed to amplify despite extensive PCR optmization and the use of additional primer pairs.

B. A Surveyor endonuclease (Cel-1) assay was performed on genomic DNA from control cells to determine whether a heterozygous SNP or a small indel were present in the region of interest. The lane number corresponds to the number of the putative off-target site in panel A. The black hatch marks on the border of each gel correspond to molecular weight marker positions (100 bp to 600 bp in 100 bp increments).

C. Results of Cel-1 assays performed on genomic DNA from 4 randomly chosen genome-edited clones. The number of the off-target site genotyped is indicated above each gel image; the lane number corresponds to the single-cell derived clone being genotyped.

Supplemental Figure 9. Experimental evaluation of genotypes in single-cell derived clones heterozygous for a ZFN-driven targeted integration event at the *PITX3* locus.

A. Table of off-target sites identified as detailed in Supplementary Methods. In the case of site OT1, the target region is extremely GC-rich, and three distinct pairs of primers failed with multiple different DNA polymerase systems to yield a specific amplicon.

B. A Surveyor endonuclease (Cel-1) assay was performed on genomic DNA from control cells to determine whether a heterozygous SNP or a small indel were present in the region of interest. The lane number corresponds to the number of the putative off-target site in panel A. The black hatch marks on the border of each gel correspond to molecular weight marker positions (100 bp to 600 bp in 100 bp increments).

C. Results of Cel-1 assays performed on genomic DNA from 4 randomly chosen genome-edited clones. The number of the off-target site genotyped is indicated above each gel image; the lane number corresponds to the single-cell derived clone being genotyped. Off-target cleavage manifests itself as a prominent doublet migrating below the primary band; cleavage was observed for off-target 5, clone #3. Off-target #10 was heterozygous for a SNP, precluding use of the Cel-1 assay. We cloned and sequenced 16 chromatids for this region from each clone; all were wild-type (data not shown).

D. Sequence of wild-type DNA from the region corresponding to off-target site 5 (see table in panel A), and of the chromatid with a single-base pair deletion observed in clone #3 (see panel C); 17 wild-type chromatids and 15 chromatids with the deletion were seen in that clone. A representative chromatogram of the latter is shown in the boxed region on the right.

Supplemental Figure 10. Experimental evaluation of genotypes in single-cell derived clones heterozygous for a ZFN-driven targeted integration event at the *OCT4* locus, ZFN pair#1.

A. Table of off-target sites identified as detailed in Supplementary Methods. The consensus used for this off-target search allowed a 1 bp gap (see supplementary methods).

B. A Surveyor endonuclease (Cel-1) assay was performed on genomic DNA from control cells to determine whether a heterozygous SNP or a small indel were present in the region of interest. The lane number corresponds

to the number of the putative off-target site in panel A. The black hatch marks on the border of each gel correspond to molecular weight marker positions (100 bp to 600 bp in 100 bp increments).

C. Results of Surveyor endonuclease assays (Cel-1) performed on genomic DNA from 4 randomly chosen genome-edited clones. The number of the off-target site genotyped is indicated above each gel image; the lane number corresponds to the single-cell derived clone being genotyped

D. Table of off-target sites identified as detailed in Supplementary Methods. The consensus used for this off-target search did not allow the 1 bp gap (see supplementary methods).

E. As panel (B) above, except off-target sites from panel (D) were used.

F. As panel (C) above, except off-target sites from panel (D) were used.

Supplemental figure 11. Experimental evaluation of genotypes in single-cell derived clones heterozygous for a ZFN-driven targeted integration event at the *OCT4* locus, ZFN pair#2.

A. Table of off-target sites identified as detailed in Supplementary Methods.

B. A Surveyor endonuclease (Cel-1) assay was performed on genomic DNA from control cells to determine whether a heterozygous SNP or a small indel were present in the region of interest. The lane number corresponds to the number of the putative off-target site in panel A. The black hatch marks on the border of each gel correspond to molecular weight marker positions (100 bp to 600 bp in 100 bp increments).

C. Results of Cel-1 assays performed on genomic DNA from 4 randomly chosen genome-edited clones. The number of the off-target site genotyped is indicated above each gel image; the lane number corresponds to the single-cell derived clone being genotyped.

Supplemental Table 1a. Targeted sequences and recognition helices of ZFNs used in this study

Supplemental Table 1b. Amino acid sequence of ZFN constructs used in this study

Supplemental Table 2. Sequences of primers used in the Cel-1 and the analysis of the Non-Targeted Allele

Supplemental Table 3.

Experimentally determined binding preferences of each ZFN used in this study as gauged via SELEX analysis[25]. Degeneracies are indicated using single letter IUPAC ambiguity codes, with K = G or T; M = A or C; Y = C or T; R = A or G; D = A, G or T; and N = any base. Note that the SELEX-derived consensus for Oct-4 ZFN#1-R is shorter than the originally targeted site, since the SELEX studies did not converge on a consensus for final six positions of its target.